REMARKS

These remarks are in response to the Final Office Action mailed November 25, 2008 and further in Response to the Advisory Action mailed June 3, 2009. No new matter is believed to have been introduced.

Applicants respectfully thank Examiner Popa and Supervisor Woitach for meeting with Applicant, Dr. Kasahara, and Applicants' representatives Dr. Jolly and Joseph Baker. At the interview Applicants explained the novelty and inventiveness of the Applicants' technology and the unexpected results achieved including increased stability through multiple passages and the transformation and spread through neoplastic cells and tissues. The parties did not reach and agreement; however, the Examiner and the Examiner's supervisor indicated that they would consider additional evidence indicating the why one would not combine the references as suggested in the Office Action as well as evidence of teaching away. Applicants have provided such evidence here and can provide additional evidence should the Examiner wish to consider additional information.

ADVISORY ACTION

In the Advisory Action mailed June 3, 2009, the Examiner indicated that the evidence presented was not entered as it was unclear why the evidence was not previously presented. Applicants respectfully submit that the evidence submitted was consistent with well accepted principles and previously set forth by Applicants in the prior responses. Furthermore, during the interview the Examiner and the Examiner's supervisor, Ex. Woitach, indicated that they would consider additional evidence in response to the Final Office Action. Applicants respectfully submit that the evidence provided is consistent with what Applicants had indicated was recognized in the art.

Applicants submit herewith additional evidence by Declaration of Dr. Yawen Chiang (See Exhibit B). As discussed during the Interview one of skill in the art would not combine the references as suggested by the Office Action nor would there be any expectation of success. Furthermore, the evidence submitted herewith comments on the unexpected results as they are recognized by an independent unbiased person of skill in the art.

The attached Declaration under 1.132 by Dr. Yawen Chiang indicates that the references cited by the Patent Office under 35 U.S.C. 103 below, although providing useful information, do not provide the motivation or expectation of success. Dr. Chiang is a co-author of peer-reviewed scientific journals with Ram et al. (see Ram et al. as cited herein) having collaborated with Ram et al. during the mid- to late-1990s and thus is familiar with the work of Ram et al. Dr. Chiang was also involved in gene therapy having been a grant reviewer and editor/reviewer for various journals. Dr. Chiang is well positioned to speak to the level of expertise, direction/motivation and results achieved in the area of gene therapy.

The Examiner will note that Dr. Chiang has stated that the invention of the present application is an advance in technology that was not expected and provides unexpected results. Furthermore, Dr. Chiang states that the references each are important for the narrow area of expertise for which they are applicable (i.e., Defective Retroviral Vectors, Herpes Viral Vectors, Avian Retroviruses, but that the references are not readily combinable as suggested in the Office Action to arrive at Applicants' claimed invention.

I. NON-STATUTORY OBVIOUSNESS-TYPE DOUBLE PATENTING

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 63-73, 75, 78-82 and 87-121 stand provisional rejected on the grounds of non-statutory obviousness-type double patenting as allegedly being unpatentable over claims 22, 23, and 26-34 of co-pending application no. 11/805,411 (the '411 application) in view of both Yan *et al.* (Prostrate, 32:129-139, 1997) and Sobol *et al.* (U.S. Patent No. 5,674,486). Applicants respectfully traverse this rejection.

Applicants respectfully submit that the rejection is moot as the '411 application is abandoned (see exhibit C).

II. REJECTION UNDER 35 U.S.C. §103

Claims 41, 43-45, 49-51, 56, 61, 66, 70, 71, 75, 78-80, 87, 89, 91, 97-102, 105, 107, 109, 115-119, and 121 stands rejected under 35 U.S.C. §103 as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of

Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486).

Claims 41, 43-45, 49-51, 56, 61, 66, 70, 71, 75, 78-80, 87, 89, 91, 97-102, 105, 107, 109, 115-121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above and further in view of Douar *et al.* (Gene Ther, 3:780-796, 1996), which is further combined to overcome certain deficiencies in the primary set of references.

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 66, 70, 71, 73, 75, 78-80, 87-92, 97-102, 105-110, 115-119, and 121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above and further combined with Vile *et al.* (Virology, 214:307-313, 1995) and Yan *et al.* (Prostrate, 32:129-139, 1997), which are further combined to overcome certain deficiencies in the primary set of references.

Claims 41, 43-45, 49-51, 56, 58, 61, 63-73, 75, 78-82, 87-119, and 121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above, and further combined with Vile *et al.* (Virology, 214:307-313, 1995) and Kasahara *et al.* (Science, 266:1373-1376, 1994), to overcome certain deficiencies in the primary set of references.

Applicants respectfully traverse these rejections as set forth previously in the Response filed August 2008, which is incorporated herein by reference. Furthermore, Applicants provide the following additional remarks, information and evidence (see, Exhibit B).

Ram *et al.* provides replication defective retroviral vectors that require a helper cell line for replication. Martuza *et al.* provides a DNA viral vector derived from Herpes Simplex Virus, a complex lytic DNA virus that induces cytotoxicity. Murakami *et al.* provides recombinant Rous Sarcoma Virus that cannot infect

mammalian cells and which comprises a disposable gene thus provided a flexible naturally inherent cassette.

Turning to the three references of Ram *et al.*, Martuza *et al.* and Murakami *et al.*, Applicants respectfully submit that one of skill in the art would not be motivated to generate Applicants' claimed invention from any combination of the foregoing references. It is important to understand that the uses, genomes and ability to infect mammalian cells are drastically different in each of the vector systems described in these references. It is not a matter of piecing together the various references as suggested by the Office Action. For example, truly piecing the references together would require placing a DNA genome in an RNA vector that can only infect avian cells and somehow making it infect mammalian cells without losing stability. As described in the prior response, one of skill in the art would not mix and match the genomes of the various vectors as suggested by the Office Action.

For example, the Examiner is directed to Exhibit A (Oh, *et al.*, J. of Virol., 76(4):1762-1768, 2002), which demonstrates that even after the priority date of the present invention, those of skill in the art would not have been motivated to generate vectors as set forth by the present claims by mixing/matching RSV and oncoretroviruses. For example, paragraph 2, column 1 at page 1762 of Oh *et al.*, states:

> Most retroviral genomes cannot accommodate the insertion of significant amounts of additional genetic information. In these cases, viral sequences must be removed to provide a place for whatever additional information is inserted. Such viruses are, by definition, replication defective. The missing viral genetic information must be supplied in *trans*, either by a helper cell or a helper virus. *There is one exception*. Avian leukosis viruses can accept approximately 2.5 kb of additional information: the naturally occurring avian leukosis virus derivative Rous sarcoma virus (RSV) contains, in addition to a full complement of viral genes, the *src* oncogene (21).
>
> (emphasis ours).

Simply put, the foregoing paragraph actually teaches away from the use of mammalian oncoretroviral vectors because they must be rendered "defective" to accommodate additional genetic material, unless you use the "one exception" an RSV vector which includes the dispensible *src* gene. This statement and the cited

reference are consistent with the remarks Applicants provided in the prior response – simply that one of skill in the art would not modify the teachings of Murakami *et al.* (regarding RSV viral vectors) to non-RSV vector systems due to the inability of such mammalian retroviral systems to accommodate additional genetic sequences.

The present application describes compositions and methods whereby this "inability" is overcome. Such an advance in the art is one of the main purposes and policies behind the patent system, i.e., to protect advances in the art and give to the inventors a limited time of exclusivity for their hard work and development.

Furthermore, turning to Martuza *et al.*, one of skill in the art would recognize that the genome of Herpes virus has little if any similarity in its genome, infectivity or life cycle to oncoretroviral vectors such as MLV. For example, Herpes virus is a lytic virus having cellular toxicity. In addition, herpes virus is far more complex and is a DNA virus. One of skill in the art would not translate the teachings in the Herpes viral arts to those of the oncoretroviral arts due to such drastic differences in genomes, infectivity and viral life cycle.

Thus, the three references of Ram *et al.*, Martuza *et al.*, and Murakami *et al.* cannot be combined without substantial changes to the genomes of the vectors described and modifications of the references that are not suggested by the art. In other words, one of skill in the art would have to discard the teachings of the references themselves to arrive at Applicants' claimed invention. For example, in order to utilize Sobol *et al.* as suggested by the Office Action, one of skill in the art would have to discard the teaching in the reference that replication competent vectors should be discarded as being dangerous. As another example, one of skill in the art would have to discard the teachings of Oh *et al.* and others in the art that oncoretroviral vectors can be modified to incorporate additional sequences even though Oh *et al.* says RSV is the "one exception".

Applicants through extensive experimentation and development demonstrate that not just any combination of elements (as suggested by the Office Action), not just any insertion site (as suggested by the Office Action) and not just any viral vector (as suggested by the Office Action) would result in Applicants' claimed invention. Applicants were the first to discover that the combination of virus selection and IRES cassette insertion site provides a competent, stable and effective RCR

system for treating cell proliferative disorders. For example, the Examiner is respectfully directed to Logg *et al.*, J. of Virol., 75(15):6989-6998, 2001, which sets forth the importance of the cassette location. The combination of transduction efficiency, transgene stability and target selectivity was unknown in any recombinant replication competent mammalian oncoretrovirus prior the instant vector. The methods (and the vector composition used in the methods) provides insert stability and maintains transcription activity of the transgene and the translational viability of the encoded polypeptide.

Applicants respectfully submit that the high-level view taken by the Office (1) fails to consider the modifications that must be made to each reference to arrive at the invention that go far beyond mere routine experimentation and (2) disregard accepted principles at the time the invention was made that replication competent retroviral vectors were unacceptable, that RSV was an "exception" to the viral vector limitations, and that HSV are not lytic and can be modified for the same purpose as the present invention. Applicants respectfully submit that the foregoing (i) lack of motivation, (ii) modifications to the references required to make them even remotely functional, and (iii) general disregard for accept principles at the time of the invention, cannot be merely discarded by a general assertion to the skill in the art.

To again put the combination of the cited references in context and to demonstrate the lack of a *prima facie* case of obviousness each reference must be explained for what it describes and then and only then can it be analyzed in combination. As will become apparent from the following remarks, the references either individually or in combination do not provide the necessary factors to set forth a *prima facie* case of obviousness.

As the Examiner indicates Ram *et al.* fail to teach or suggest a recombinant replication competent oncoretroviral vector or recombinant plasmid or recombinant polynucleotide encoding a replication competent oncoretroviral vector. Furthermore, Ram *et al.* fail to teach or suggest treating a tumor in the absence of a helper cell to assist in the defective viral replication, Ram *et al.* fails to teach or suggest a cytokine transgene, Ram *et al.* fails to teach or suggest a chimeric env protein, Ram *et al.* fails to teach or suggest a tissue specific promoter, and Ram *et al.* fails to teach or suggest an IRES cassette. Ram *et al.* is far removed from the methods and

compositions of Applicants' invention, which utilize a replication competent, non-helper cell system to treat a cell proliferative disease or disorder.

The cited reference of Ram *et al.* describes a method that utilizes "retroviral producer cells" injected at the site of a tumor (see page 86, column 2, last paragraph of the cited reference). The producer cells support the *in situ* production of a retroviral vector containing a suicide gene. The producer cells are necessary because the vector is not replication competent. Further, the nucleic acid sequence encoding the suicide gene is located "just downstream of the 5' long terminal repeat sequence" (see page 84, column 1, lines 2-4 of the cited reference) in a location different from Applicants' claimed vector. It is clear from the contents of the cited reference that Ram *et al.* fail to appreciate the significance of utilizing a replication competent oncoretrovirus in the absence of a producer cell to achieve efficient transduction. Because Ram *et al.* use a gutted vector in order to incorporate the transgenes (see, Oh *et al.* described above), transcription of a transgene can easily be effected off the regulatory region of the 5'LTR. In contrast, Applicants' transgene is not directly linked to the 5'LTR. The location of the transgene and the IRES as set forth in Applicants' claims is not an insignificant modification.

Thus, Ram *et al.* is deficient in at least three aspects: (1) the vector is replication defective; (2) the methods require a help cell; and (3) the transgene location is of little or no important to Ram *et al.* The gutted size and location of the transgene in Ram *et al.* allow for the 5' LTR to serve as the regulatory region. To overcome these deficiencies the Office combines Ram *et al.* with Martuza *et al.*

The cited reference of Martuza *et al.* allegedly teach replication competent viral vectors derived from adenovirus and herpes simplex virus (such vectors are DNA vectors - very different than RNA vectors). Applicants respectfully submit this is the first of many leaps the Office makes to overcome voids in the development of Applicants' claimed invention. First, it is not clear why one would combine a defective retrovirus of Ram *et al.* with a DNA virus of Martuza *et al.*, the genomes are completely different. Nevertheless, when the references are combined the combination still fails to teach or suggest Applicants' claimed invention. Like Ram *et al.*, Martuza *et al.* fail to appreciate the importance of positioning a heterologous sequence encoding a therapeutic polypeptide in a region outside the LTR or not

linked directly to the LTR of the viral vector. Nor does the combination of references teach, suggest, or appreciate an internal ribosome entry site. As will be recognized by the Examiner and those of skill in the art, merely inserting a transgene into a replication competent retrovirus does not provide a reasonable expectation that infectivity, stability or continued transmission and expression of the transgene will occur. In fact, numerous peer-reviewed journal articles indicate that insertion of transgene into U3 and other locations within a replication competent retrovirus can cause a loss of replication, and genetic instability of the vector (see, e.g., Logg *et al. supra*). Thus, the combination of Ram *et al.* and Martuza *et al.* fail to teach or suggest Applicants' claimed invention and do not provide any reasonable expectation of success in achieving a RCR having the transmission and genetic stability of Applicants' claimed vectors.

To overcome the deficiencies of Ram *et al.* and Martuza *et al.*, the Office combines Murakami *et al.*

Murakami *et al.* use a Rous Sarcoma Virus. As stated by Oh *et al.* (Exhibit A), the RSV is the one "exception" to retroviruses. The IRES-transgene insertions described in Murakami *et al.* consist of an IRES-transgene sequence positioned 3' to the env-encoding sequence and 5' to the 3' LTR. However, the cited reference utilizes replication competent avian sarcoma viruses (RCAS) which are distinct from the oncoretroviruses of the pending claims and incapable of replication in mammalian cells. Thus, the RSV vector could not be used to treat a mammal as set forth in Applicants' claims. The inability of RSV to produce infective viral particles in mammalian cells is disclosed in several peer-reviewed journal articles. Here, again, the Office makes a leap from a defective gutted retroviruses, to DNA viruses to avian viruses, with little direction, suggestion or likelihood of success in the art particularly when the RSV virus is recognized as an exception for its ability to incorporate transgenes into its genome. It is only through Applicants' disclosure and hindsight reconstruction that such very different viral architectures and functions can be pieced together. For example, Avian Rous Sarcoma Virus naturally carries extra sequences (the src oncogene, which is in addition to the gag, pol and env genes required for replication, and which is similar in size to the env gene) positioned just after env. Thus, RSV evolved a capacity to incorporate a large piece of extra sequence in this

location in its genome, something not found in mammalian oncoretroviruses. The idea of putting an IRES-transgene insert after the *env* gene in a mammalian oncoretrovirus would not be obvious in view of the cited references simply because there are no known naturally-evolved replication-competent mammalian oncoretroviruses with extra genes following the *env* (or anywhere else, for that matter). In fact, it was recognized in the art that inserting a transgene in the region following the env gene although providing short term expression ultimately resulted in genetic instability and loss of the transgene in subsequent rounds of replication. RSV through natural development has developed a "transgene insertion site" because it contained a non-essential and replaceable gene (*src*), thus providing additional flexibility (i.e., an "exception") compared to mammalian oncoretroviruses.

Further, combining the IRES-transgene of Murakami *et al.* and the vector described by Martuza *et al.* would not result in a vector or method described or claimed in the instant application. It is not clear why or how one would combine a DNA viral vector and an RNA avian viral vector.

Finally, the Office combines Sobol *et al.* with Ram *et al.*, Martuza *et al.*, and Murakami *et al.*, for the teaching of cytokines to treat cancer. The Office appears to be picking and choosing the use of certain reference and avoiding the teachings of the reference as a whole. When taken as a whole, Sobol *et al.* actually teach that one should avoid the use of replication competent retroviruses. For examples, Sobol *et al.* teach throughout the specification that one should use proper screening, production and removal of replication competent retroviruses from any system or method. However, even in view of such a teaching away, Sobol *et al.* do not remedy the deficiencies as set forth above regarding the replication competent retrovirus and use of such recombinant viral vectors for the treatment of cell proliferative disorder.

Not only do the cited references when combined fail to identify predictable solutions for achieving a replication competent oncoretrovirus capable of delivering a therapeutic polypeptide to dividing cells, they also fail to provide all the components necessary for the production of the vector set forth in the claimed methods.

In contrast, the Applicants have succeeded in developing a replication competent oncoretroviral vector with an enhanced capability to stably deliver a heterologous sequence to a dividing mammalian cell. Once integrated into a target

cell, the novel vector produces a therapeutic polypeptide encoded by the heterologous sequence. In addition, viral particles which infect neighboring dividing cells are also produced in the absence of helper cells.

It is important to understand that the surprising combination of transduction efficiency, transgene stability, and target selectivity provided by Applicants' inventions were simply unknown in any recombinant replication competent mammalian oncoretrovirus prior to the Applicants' invention. When placed in a mammalian oncoretroviral background, the cassette is useful for the stable expression of a transgene coding sequences including marker genes such as green fluorescent protein (GFP), suicide genes such as thymidine kinase, cytosine deaminase (CD) or purine nucleoside phosphorylase (PNP), and genes encoding cytokines such as interferon.

For at least the foregoing reasons, the pending claims are novel and non-obvious over the cited reference. Accordingly, Applicants respectfully request withdrawal of this rejection.

III. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 63-73, 75, 78-82, and 87-121 stand rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. In particular, the Office Action alleges that the term, "at the 5' or 3' end" is new matter. Applicants respectfully traverse this rejection.

As set forth previously, Applicants respectfully direct the Examiner to page 64, lines 16-20, which explains that the LTR need only be present at one or both of the 5' or 3' ends and that during reverse transcription the LTR is duplicated. Accordingly, delivery of a vector comprising a single LTR would be sufficiently duplicated during the RCR life cycle (see also page 67 and figure 8). Furthermore, page 18 and 19 explain the viral life cycle including that upon transcription the provirus "now" has two identical repeats at either end. The use of "now" indicates that it did not previously have LTRs at both ends but only after transcription of the provirus does it "now" have LTRs at both ends.

However, to advance prosecution and without disclaimer, and in order to place the case in better form for appeal, Applicants have amended the claims to remove "5' or 3'".

For at least the foregoing, the Applicant submits that the claimed invention is patentable and request reconsideration and notice of such allowable subject matter.

The Director is authorized to charge any required fee or credit any overpayment to Deposit Account Number 50-4586, please reference the attorney docket number above.

The Examiner is invited to contact the undersigned at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted,

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EXHIBIT A

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Construction and Characterization of a Replication-Competent Retroviral Shuttle Vector Plasmid

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We constructed two versions of an RCASBP-based retroviral shuttle vector, RSVP (RCASBP shuttle vector plasmid), containing either the zeocin or blasticidin resistance gene. In this vector, the drug resistance gene is expressed in avian cells from the long terminal repeat (LTR) promoter, whereas in bacteria the resistance gene is expressed from a bacterial promoter. The vector contains a bacterial origin of replication (ColE1) to allow circular viral DNA to replicate as a plasmid in bacteria. The vector also contains the *lac* operator sequence, which binds to the *lac* repressor protein, providing a simple and rapid way to purify the vector DNA. The RSVP plasmid contains the following sequence starting with the 5′ end: LTR, *gag*, *pol*, *env*, drug resistance gene, *lac* operator, ColE1, LTR. After this plasmid was transfected into DF-1 cells, we were able to rescue the circularized unintegrated viral DNA from RSVP simply by transforming the Hirt DNA into *Escherichia coli*. Furthermore, we were able to rescue the integrated provirus. DNA from infected cells was digested with an appropriate restriction enzyme (*Cla*I) and the vector-containing segments were enriched using *lac* repressor protein and then self-ligated. These enriched fractions were used to transform *E. coli*. The transformation was successful and we did recover integration sites, but higher-efficiency rescue was obtained with electroporation. The vector is relatively stable upon passage in avian cells. Southern blot analyses of genomic DNAs derived from successive viral passages under nonselective conditions showed that the cassette (drug resistance gene-*lac* operator-ColE1) insert was present in the vector up to the third viral passage for both resistance genes, which suggests that the RSVP vectors are stable for approximately three viral passages. Together, these results showed that RSVP vectors are useful tools for cloning unintegrated or integrated viral DNAs.

The retroviral life cycle depends on the conversion of the RNA genome found in virions into DNA and the subsequent integration of the DNA into the host cell genome. There are a number of different types of experiments for which the molecular cloning of either unintegrated or integrated viral DNA is a critical step. In most cases, cloning the viral DNA involves standard recombinant DNA techniques: viral DNA is inserted into a plasmid or a prokaryotic viral vector. However, there is an alternative approach. It is possible to introduce elements into retroviral vectors that allow DNA forms of the viral genome to replicate in prokaryotic hosts. These types of vectors are referred to as shuttle vectors and can simplify the recovery of viral DNA. At a minimum the shuttle vector must have a plasmid origin of replication and a gene (promoter and coding region) that can be selected in a bacteria.

Most retroviral genomes cannot accommodate the insertion of significant amounts of additional genetic information. In these cases, viral sequences must be removed to provide a place for whatever additional information is inserted. Such viruses are, by definition, replication defective. The missing viral genetic information must be supplied in *trans*, either by a helper cell or a helper virus. There is one exception. Avian leukosis viruses can accept approximately 2.5 kb of additional information: the naturally occurring avian leukosis virus derivative Rous sarcoma virus (RSV) contains, in addition to a full complement of viral genes, the *src* oncogene (21). We have prepared a family of replication-competent retroviral vectors, collectively called the RCAS vectors, that derive from the Schmidt-Ruppin strain of RSV. Basically, in the RCAS vectors, the *src* gene has been deleted and a unique restriction site has been left at the site of the deletion to simplify the insertion of foreign DNA. To permit the cloning and amplification of the RCAS vector DNA, the viral genome was linked to pBR322-derived plasmid sequences (10); however, the plasmid sequences lie outside the viral replicon and the viral vector brings none of the prokaryotic plasmid sequences with it when it replicates in avian cells. It is possible to insert prokaryotic plasmid sequences into the *Cla*I site of an RCAS vector. The following two versions have been created: p779NC327AC28F, which contains a pBR origin and an ampicillin resistance gene (unpublished observations), and a derivative, pANV-A, which contains a pBR origin, a simian virus 40 promoter, and a neomycin resistance gene linked to the Tn*5* promoter (15).

The p779NC327AC28F plasmid has two disadvantages. First, there is no selection possible when the virus is propagated in avian cells. Second, the prokaryotic sequences are rapidly lost during virus propagation (unpublished observations). pANV-A can be selected in both prokaryotic and eukaryotic hosts, and the viral genome was reported to be stable through one round of viral replication (15).

We have revisited the RCAS shuttle vector problem and have prepared two new vectors that have advantages over the published vectors. First, the new vectors make use of drug resistance markers that are small (zeocin resistance and blasticidin resistance). Zeocin is a member of the bleomycin/phleomycin family of antibiotics and is a basic, water-soluble, copper-chelated glycopeptide isolated from *Streptomyces verticillus*

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(2). When zeocin enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{+} and zeocin becomes activated. Zeocin will then bind DNA and cleave it, causing cell death (2). A zeocin resistance protein, the product of the *Streptoalloteichus hindustanus ble* gene, has been isolated from *S. hindustanus*, binds to zeocin, and inhibits its DNA cleavage activity (4, 6). The *S. hindustanus ble* gene has been used as a selectable marker for prokaryotes and eukaryotes (3–5, 14). Blasticidin S is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* that is a potent protein synthesis inhibitor for both prokaryotes and eukaryotes (24, 25). Blasticidin resistance is conferred by the expression of one of two blasticidin S deaminase genes, either the *BSD* gene of *Aspergillus terreus* (12) or *bsr* of *Bacillus cereus* (11). These enzymes convert blasticidin S to an inactive deaminohydroxy derivative (11). In eukaryotic cells, the RSVP vectors express the drug resistance genes via a spliced message, so that an internal promoter is not needed. In addition, we have added a completely symmetric *lac* operator, which makes it a simple matter to enrich for viral DNA (16, 17). This symmetric *lac* operator, which is an inverted repeat of a 15-bp segment from the left half of the natural operator sequence (5′-TGTGGAATTGTGAGCGCTCACAATTCCACA-3′), has been reported to bind the *lac* repressor 10-fold more tightly than the natural *lac* operator sequence (19). It is possible with this system not only to recover unintegrated circular viral DNA from infected cells but also to easily clone integration sites. The blasticidin and zeocin cassettes are approximately 1.2 kb long, so it should be possible to introduce additional information into the shuttle vectors. We have also demonstrated that the RSVP vectors are sufficiently stable that they can be passaged three times without a substantial loss of the prokaryotic plasmid sequences.

MATERIALS AND METHODS

Plasmid construction. A cassette containing multiple cloning sites (MCS), a drug resistance gene (either zeocin or blasticidin), a *lac* operator sequence, and a ColE1 replication origin was introduced into the plasmid 327AC (10) as described below. The plasmid 327AC was digested with *Eco*RI and *Hin*dIII, and the digested DNA was ligated with two complementary 40-bp oligonucleotides that included the following cloning sites: *Cla*I, *Bgl*II, *Avr*II, *Not*I, *Xba*I, and *Hin*dIII. The resulting plasmid was called 327ACMC. The blasticidin resistance gene was PCR amplified from the pcDNA6/V5-His/*lac*Z plasmid (InVitrogen, Carlsbad, Calif.) by using the forward primer Bsd-Not, which anneals to the region upstream of the EM-7 promoter, and the reverse primer Bsd-Xba, which spans the termination codon for the blasticidin resistance gene. The sequence of Bsd-Not was 5′-ATCAgcggccgcATCAGCACGTGTTGAC-3′. The Bsd-Not primer contains a *Not*I restriction site in the overhang, which is indicated by lowercase letters. The sequence of Bsd-Xba was 5′-ACtctagaTTAGCCCTC CCACACATAACC-3′. The *Xba*I restriction site is indicated by lowercase letters, and the stop codon is underlined. The PCR product was cleaved with *Not*I and *Xba*I and inserted into the vector 327ACMC that had been digested with *Not*I and *Xba*I, which generated 327(Bsd). Next, two cDNA oligonucleotides (LacO-Xba, CTAG**ATGTGGAATTGTGAGCGCTCACAATTCCACA**ggatccTA; LacO-Hind, AGCTTAA**ggatcc****TGTGGAATTGTGAGCGCTCACAATTCCACA** T), containing the *lac* operator (bold) and a new *Bam*HI site (lowercase), were annealed and inserted into the *Xba*I/*Hin*dIII site of 327(Bsd), which generated 327(Bsd/Lac). The *lac* operator was sequenced to check its integrity. To create unique MCS, two cDNA oligonucleotides (MCS-Cla, CGATACTAGTCGTAC GATGCATGC; MCS-Not, GGCCGCATGCATCGTACGACTAGTAT), containing *Spe*I, *Sph*I, and *Nsi*I sites, were annealed and inserted into the *Cla*I/*Not*I site of 327(Bsd/Lac), which generated 327(MCS/Bsd/Lac). The ColE1 origin in the cassette was PCR amplified from the pcDNA6/V5-His/*lac*Z plasmid (InVitrogen) using the forward primer Ori-*Bam*H (TTCGGATCCATGTGAGCA AAAGGCCAGCAA) and the reverse primer Ori-Hind (GTCAAGCTTacgcgt CCCGTAGAAA AGATCAAAGGA). The reverse primer created an *Mlu*I site (lowercase letters) which was used in subsequent cloning steps. The PCR product was cleaved with *Bam*HI and *Hin*dIII and inserted into the *Bam*HI/*Hin*dIII site of 327(MCS/Bsd/Lac). The resulting plasmid was called 327ca(Bsd).

The zeocin resistance gene with the associated EM-7 promoter was PCR amplified from the pZeoSV2(+) plasmid (InVitrogen) using two primers, Zeo-Not (ATTgcggccgcTGTTGACAATTAATCATCGGC) and Zeo-Xba (GCCtcta gaTCAGTCCTGCTCCTCGGCCAC). A *Not*I site (lowercase) in the Zeo-Not primer was introduced upstream of the EM promoter and an *Xba*I site (lowercase) in the Zeo-Xba primer was introduced immediately downstream of the stop codon (underlined). The resulting PCR fragment was digested with *Not*I and *Xba*I and used to replace the *Not*I/*Xba*I fragment containing the blasticidin resistance gene of 327ca(Bsd), resulting in the plasmid 327ca(Zeo).

The retroviral backbone was constructed in the plasmid 779/2795 (9). To insert the *pol* region from the Bryan strain of RSV, a 4.7-kb *Sac*I-to-*Kpn*I fragment containing the *gag-pol* region of 779/2795 was replaced with a *Sac*I-to-*Kpn*I fragment of RCASBP(A) (18), which generated the plasmid 779BP. The cassette described above was purified as a *Cla*I-to-*Mlu*I fragment from 327ca(Bsd) and 327ca(Zeo) and was inserted into the *Cla*I-to-*Mlu*I site of 779BP to generate 779BP-ca(Bsd) and 779BP-ca(Zeo), respectively. These vectors, however, contained an ampicillin resistance gene and a second replication origin derived from pBR322 that was present in the original 779/2795 plasmid. The *Sal*I fragment containing both the ampicillin gene and the second origin was removed from the 779BP-ca(Bsd) and 779BP-ca(Zeo) plasmids to generate 779(ΔSal)BP-ca(Bsd) and 779(ΔSal)BP-ca(Zeo) plasmids. Finally, to introduce a 3′ splice acceptor site upstream from the drug-resistant genes in these vectors, the splice acceptor-containing segment was taken from RCASBP(A) as a *Sal*I-*Cla*I fragment. This fragment was used to replace the corresponding *Sal*I-*Cla*I segment in 779(ΔSal) BP-ca(Bsd) and 779(ΔSal)BP-ca(Zeo), which generated RSVP(A)B and RSVP (A)Z, respectively.

Cells, transfection, and infection. DF-1, a continuous line of chicken fibroblasts, was derived from EV-O embryos (7, 20). The cells were maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 5% fetal bovine serum, 5% newborn calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin (Quality Biological, Inc., Gaithersburg, Md.) per ml and incubated at 39°C with 5% CO_2. Cells were passaged 1:3 at confluence with trypsin DeLarco (pH 6.8). Plasmid DNA was introduced into DF-1 cells by calcium phosphate precipitation (13). Precipitates containing 10 μg of DNA per 100-mm-diameter plate were incubated with subconfluent DF-1 cells for 4 h at 39°C and then with medium containing 15% glycerol for 5 min at 39°C. Cells were washed twice with phosphate-buffered saline and incubated in growth medium for 48 h. The transfected cells were passaged two to three times to let the viruses spread throughout the culture. Culture medium containing the virus was harvested and subjected to low-speed centrifugation to remove cellular debris. A portion of the infectious virions was used to infect fresh DF-1 cells. Selection for drug resistance was initiated at 48 h postinfection at 10 μg/ml for blasticidin (InVitrogen) and 400 μg/ml for zeocin (InVitrogen).

Cloning unintegrated viral DNA into *Escherichia coli*. The circularized unintegrated viral DNA was recovered from infected cells by the method of Hirt (8). Three hundred nanograms of the recovered DNA was used to transform chemically competent *E. coli* DH5α (Life Technologies, Gaithersburg, Md.) or ElectroMax DH10B (Life Technologies) by electroporation. Electroporation was performed with the BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.). Recipient cells were subjected to a single 5-ms pulse at a field strength of 5.5 kV/cm using a 2-mm gap Gene Pulser cuvette electrode (Bio-Rad, Hercules, Calif.) at room temperature. After a 1-h recovery period in NZY broth at 37°C, the transformed bacteria were plated onto low-salt Luria-Bertani plates containing either 100 μg of blasticidin per ml or 25 μg of zeocin per ml.

***lac* repressor-mediated recovery of integrated retroviral DNA.** *lac* repressor-mediated recovery was carried out essentially as described previously (16, 17). Either 200 or 100 μg of genomic DNA from the RSVP(A)B-infected DF-1 cells or RSVP(A)Z-infected DF-1 cells, respectively, was digested with *Cla*I. The reaction mixture was adjusted to 150 mM NaCl, 10 mM EDTA, 50 μg of bovine serum albumin per ml, and 10% (vol/vol) glycerol in a final volume of 600 μl. The digested DNA was incubated with 6 μg of *lac* repressor protein (kindly provided by P. Lu) for 30 min at room temperature. The DNA-*lac* repressor protein mixture was then filtered through nitrocellulose which had been pretreated with 0.4 M potassium hydroxide and washed twice with water. The nitrocellulose filter was washed three times with 1.5 ml of wash buffer (150 mM NaCl, 10 mM EDTA) and eluted twice with 1 ml of elution buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 10 mM isopropyl-β-D-thiogalactopyranoside [IPTG]) for 30 min at 37°C. The enriched DNA was extracted with phenol-chloroform and precipitated with ethanol. The precipitated DNA was ligated with the Rapid DNA

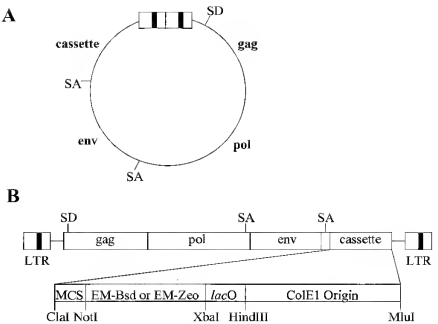

FIG. 1. Schematic drawing showing the structure of the RSVP vector. (A) RSVP. The viral genes *gag*, *pol*, and *env* are shown (not to scale). The positions of the splice donor (SD) and SA are also shown. (B) Schematic representation of the cassette. EM-Bsd, EM-7 promoter-blasticidin resistance gene; EM-Zeo, EM-7 promoter-zeocin resistance gene; *lacO*, *lac* operator.

ligation kit (30 U/200 μl; Roche, Indianapolis, Ind.) for 18 h at 16°C. The ligated DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 8 μl of water. Two microliters of DNA was used for transformation as described above.

Sequencing of the integrated viral DNA. DNA sequences were determined by cycle sequencing with a primer specific for the U5 region of RCASBP using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing reactions were analyzed with an automated 373A DNA sequencer (Applied Biosystems). The sequence of the primer was 5′-ACCACATTGGTGTGCACCTGG-3′.

Southern blot. Genomic DNA was isolated from the infected cells using the cell culture DNA Midi kit (Qiagen, Valencia, Calif.) following the manufacturer's recommendations. For Southern blot analyses, 15 μg of genomic DNAs was digested with an appropriate restriction enzyme (*Eco*RI) and separated by electrophoresis on 1% agarose–Tris-acetate-EDTA buffer gels. DNA samples were denatured with alkali, neutralized, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by capillary blotting with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were baked at 80°C under vacuum for 2 h. The hybridization probe (encompassing *env* to the long terminal repeat [LTR] region) was a 1.2-kb *Eco*RI fragment purified from RCASBP(A) and labeled with [α-^{32}P]dCTP using Prime-It II (Stratagene, La Jolla, Calif.). Prehybridizations and hybridizations were carried out overnight at 42°C. Hybridization solution contained 50% formamide, 1× Denhardt solution, 0.5% sodium dodecyl sulfate, 7.5% dextran sulfate, and 5× SSC. After stringent washes, membranes were autoradiographed for 1 day.

RESULTS

Construction of replication-competent RSVP. We constructed RSVP by inserting a cassette (MCS-drug resistance gene-*lac* operator-ColE1) into the replication-competent avian retroviral vector 779BP. 779BP is a derivative of 779/2795 that was constructed by replacing the *pol* region of 779/2795 with the corresponding *pol* region of the Bryan high-titer strain of RSV (18, 23). In terms of the retroviral vector portion carried by the plasmid, 779/2795 is the same as RCAN(A) and 779BP is the same as RCANBP(A). The details of this construction are given in Materials and Methods. The final form of the plasmids is shown in Fig. 1. The cassette contains one of two drug resistance genes, either blasticidin or zeocin resistance. The RSVP vectors contain a splice acceptor (SA) sequence immediately upstream of the cassette. The drug resistance gene is expressed in avian cells as a subgenomic mRNA from the viral promoter within the LTR, whereas in bacteria the same gene is expressed from the EM-7 bacterial promoter. The EM-7 promoter is synthetic and was isolated from the plasmids carrying the blasticidin and zeocin resistance genes (see Materials and Methods). To provide a simple and rapid purification of the vector DNA, the perfectly symmetric *lac* operator sequence was inserted in the cassette. Finally, the cassette contains a bacterial origin of replication (ColE1) to allow closed circular forms of viral DNA to replicate as plasmids in bacteria.

Recovery of unintegrated viral DNA in *E. coli*. To rescue unintegrated retroviral DNA, the RSVP vectors were transfected into DF-1 cells and the transfected cells were passaged two times to let the viruses spread throughout the culture. RSVP(A)Z virus confers resistance to zeocin and the RSVP(A)B virus confers resistance to blasticidin. Because the viruses are replication competent, it was not possible to determine the titer in DF-1 cells. However, the RSVP vectors appear to replicate as efficiently after transfection (or infection) as the corresponding RCASBP(A) vectors. The viral supernatant was harvested and used to infect fresh DF-1 cells. To show that unintegrated viral DNAs present in these infected DF-1 cells were due to the successful viral infection and not because of carryover of RSVP plasmids from the transfected DF-1 cells, the circularized unintegrated retroviral DNAs were obtained from infected cells by the method of Hirt. The DNA was used to transform *E. coli*, selecting either blasticidin- or zeocin-resistant colonies. To demonstrate that the viral DNAs rescued from infected cells contained both of the circular forms of viral DNA (one-LTR and two-LTR circles), restriction digests were carried out. The starting plasmid, used to initiate infection, was a two-LTR circle. The only *Sac*I site in RSVP is located between the LTR and the *gag* gene, and the *Cla*I site is between *env* and the LTR. Thus, digestion with *Cla*I and *Sac*I should yield two fragments, a 6.8-kb vector DNA fragment and either a 2.1-kb fragment if the proviral DNA contains two LTRs or a 1.8-kb fragment if it contains one LTR (Fig. 2A). For RSVP(A)B, of the 13 recovered plasmids, 4 contained only one LTR, 2 contained two LTRs (Fig. 2B), and 7 were the products of autointegration events (data not shown) (15, 22). Similarly, for RSVP(A)Z, of the 20 recovered plasmids, 10 contained one LTR, 3 contained two LTRs (Fig. 2C), and 7 were autointegration products (data not shown). This result suggests that proviral DNAs observed in infected cells were due to successful replication of RSVP RNA and transfer of viral particles and not due to carryover of RSVP from the initial transfection.

Although we were able to get appropriate clones, the recovery was relatively low with chemically competent cells. We tried using electroporation to increase the transformation efficiency (see Materials and Methods for details). When the same amount of DNA sample was used to transform *E. coli* by electroporation, we obtained 238 and 802 transformants from RSVP(A)B and RSVP(A)Z, respectively. These results suggest that the efficiency of unintegrated plasmid recovery was 15 to 40 times higher with electroporation.

Rescue of integrated viral DNA. To rescue integrated viral DNA, genomic DNA was isolated from infected cells and digested with *Cla*I. Since one *Cla*I site comes from the RSVP

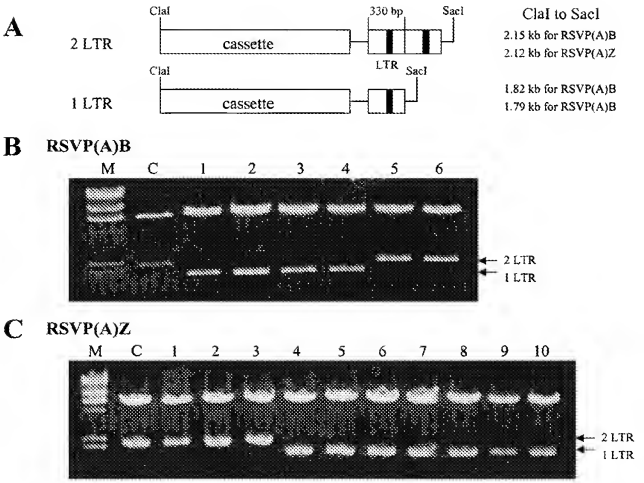

FIG. 2. Rescue of unintegrated retroviral DNA. Low-molecular-weight DNA was isolated by HIRT extraction and the extracted DNA was used to transform *E. coli* DH5α. The selection was for either blasticidin- or zeocin-resistant colonies. (A) Diagram showing the difference between two-LTR and one-LTR DNA segments. (B and C) Plasmids were digested with *Cla*I and *Sac*I. The positions of DNAs containing two LTRs and one LTR are indicated. C, parental RSVP vector; M, size marker (λ DNA digested with *Hin*dIII).

vector, the second *Cla*I site must come from the adjacent cellular DNA (Fig. 3A). The *Cla*I-digested DNAs were enriched for viral sequences by binding to the *lac* repressor protein (see Materials and Methods) and then self-ligated. These enriched fractions were used to transform *E. coli*. Restriction cleavage analyses with *Cla*I and *Mlu*I showed that 3 of the 16 RSVP(A)B transformants and 3 of the 7 RSVP(A)Z transformants contained cellular DNA (Fig. 3B). For these plasmids, double digestion with *Cla*I and *Mlu*I generated a band representing the cassette insert. In addition, the digestion yielded

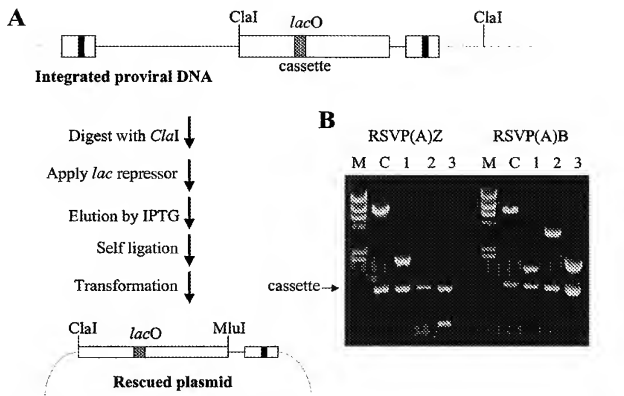

FIG. 3. Rescue of integrated retroviral DNA. (A) Schematic diagram of *lac* repressor-mediated recovery (see Materials and Methods for details). (B) Plasmids were digested with *Cla*I and *Mlu*I. The position of the cassette insert is indicated. C, parental RSVP vector; M, size marker (λ DNA digested with *Hin*dIII).

```
                    LTR-U5 ▼ LTR-U3
RSVP        ...AAGGCTTCATTAATGTAGTCTT...
RSVP(A)B #1 ...AAGGCTTCATTTGGTGACCAGA...
RSVP(A)B #2 ...AAGGCTTCAGACAGGTGTAGTT...
RSVP(A)B #3 ...AAGGCTTCAAGAAGATTCAGAA...
RSVP(A)Z #1 ...AAGGCTTCAGCCTTGATTTATT...
RSVP(A)Z #2 ...AAGGCTTCATTAAGACAGCTAT...
RSVP(A)Z #3 ...AAGGCTTCACCATTTTTAAGAT...
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FIG. 4. Sequences of integrated retroviral DNA. The inverted triangle indicates the boundary between the two LTRs. Sequences flanking the LTR are shown in bold.

another band of variable size due to the variable position of the nearest *Cla*I cleavage site in the cellular DNA. To demonstrate whether these inserts were derived from the cellular DNA, rescued plasmids were directly sequenced with a primer derived from the U5 sequence of the viral vector. As expected, all plasmids showed the integration-specific sequence feature that a CA dinucleotide pair was present at the site of joining to host DNA (Fig. 4). RSVP(A)B clones 2 and 3 and RSVP(A)Z clones 1 and 3 appear to contain novel inserts derived from the cellular DNA. However, sequencing results revealed that RSVP(A)B clone 1 and RSVP(A)Z clone 2 contained inserts identical to part of the RSVP vector sequence, indicative of autointegration (22).

As described above, we tried electroporation to obtain additional integration sites. When the same amount of enriched DNA sample was used to transform *E. coli* by electroporation, we were able to get 1,760 and 886 transformants from RSVP (A)B and RSVP(A)Z, respectively; thus, the efficiency of enriched plasmid recovery was more than 100 times higher with electroporation.

When we recovered integrated viral DNA, in addition to the plasmids containing integration sites we also obtained plasmids of approximately 3 kb in more than half of the clones. Further analyses of these plasmids revealed that they were by-products derived from the circularized unintegrated viral DNAs. There is a second *Cla*I site within the *gag* gene (Fig. 5A). In plasmids grown in *E. coli,* this site is subject to dam methylation. However, there is no dam methylation in avian cells. When genomic DNA isolated from the infected DF-1 cells was digested with *Cla*I, both *Cla*I sites in the circular unintegrated viral DNAs were cleaved and the cassette (*lacO*)-containing DNA segment was enriched by binding to the *lac* repressor protein. These 3-kb plasmids were generated by self-ligation and were recovered by transformation into *E. coli.* After transformation and growth in *E. coli,* the *Cla*I site is again subject to dam methylation and thus digestion with *Cla*I did not cut the 3-kb mini plasmids. Digestion with *Mlu*I cut the mini plasmids once and digestion with *Bam*HI generated two fragments, of 1.54 and 1.39 kb, as expected (Fig. 5B). The recovery of the 3-kb mini plasmid provides additional evidence that the unintegrated viral DNAs were generated by successful viral infection rather than carryover of initial RSVP plasmids from the transfected DF-1 cells.

Stability of vectors. To determine that the cassette insert is reasonably stable upon viral passage in avian cells, genomic DNAs derived from virus that had been passaged under non-

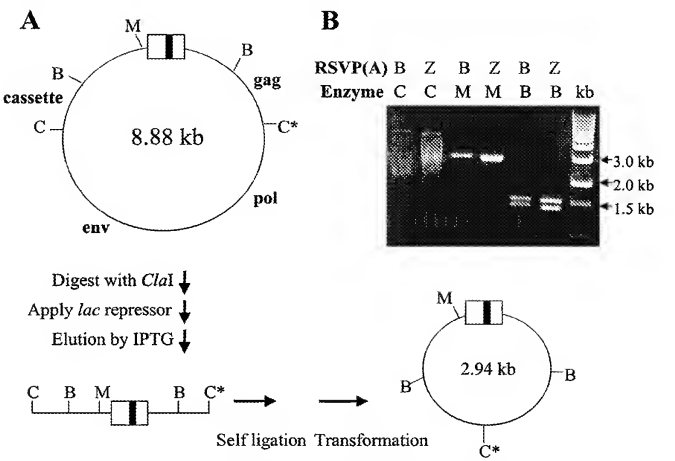

FIG. 5. Major by-product obtained in the recovery of integrated viral DNA. (A) Schematic drawing of the generation of the plasmid. M, *Mlu*I; B, *Bam*HI; C, *Cla*I; C*, *Cla*I site within *gag* gene subjected to dam methylation in *E. coli* but not in avian cells. (B) Restriction enzyme analyses of the plasmid. Digestion with *Cla*I did not cut the plasmid due to dam methylation. Digestion with *Mlu*I cut the plasmid once and digestion with *Bam*HI generated two fragments, of 1.54 and 1.39 kb, as expected. kb, DNA ladder in kilobases.

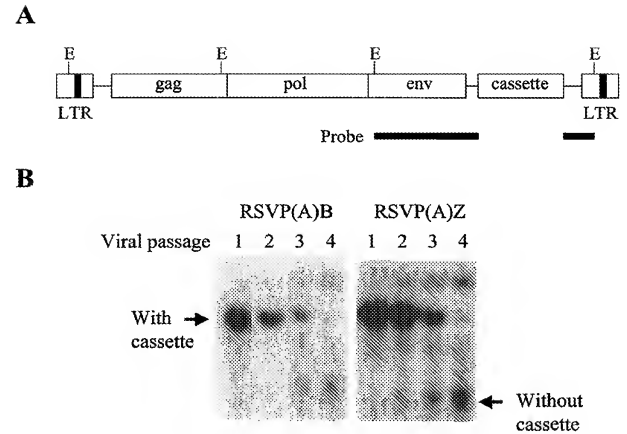

FIG. 6. Southern transfer analyses of the stability of the RSVP vector. (A) The probe was prepared from RCASBP(A) as a 1.2-kb *Eco*RI fragment to provide equal opportunity to hybridize to *Eco*RI fragments that contained (or had lost) the cassette. *Eco*RI recognition sites are indicated (E) (not to scale). (B) Detection of the cassette insert in the genomic DNA derived from cells infected with the RSVP(A)B and RSVP(A)Z vectors. Genomic DNA was digested with *Eco*RI, resolved in an agarose gel, transferred onto a nitrocellulose membrane, and hybridized with ^{32}P-labeled DNA prepared from the 1.2-kb *Eco*RI fragment of RCASBP(A). The larger band represents a 2.4-kb *Eco*RI fragment containing the insert, whereas the smaller band represent a 1.2-kb *Eco*RI fragment lacking the insert.

selective conditions were digested with the restriction enzyme *Eco*RI and analyzed by Southern blot. To provide equivalent hybridization to *Eco*RI fragments that contained the cassette and the related fragments from viral genomes that had lost the cassette, the probe was prepared from RCASBP(A) as a 1.2-kb *Eco*RI gel-purified DNA fragment (Fig. 6A). As can be seen in Fig. 6B, the cassette insert was present in the vector up to three viral passage for both drugs, but there was significant loss in the fourth viral passage, suggesting that RSVP vectors are stable for approximately three viral passages. The efficiency of transformation does affect the apparent stability of the insert; a relatively efficient transformation gives a larger burst of virus and fewer rounds of replication are required to get the virus stock. With this caveat, the stability of the two drug cassettes are similar to an "average" insert in RCASBP(A).

DISCUSSION

We have developed two related replication-competent retroviral shuttle vectors (RSVPs). The sequences that permit the viral DNA to replicate as a plasmid and to be selected in *E. coli* (either blasticidin resistance or zeocin resistance) are small (approximately 1.2 kb), which should make it possible to insert additional sequence in these vectors. The drug resistance genes were inserted in a fashion that permits expression of the selectable markers (via a spliced message) in avian cells; as expected, infection with the vectors confers resistance. The inserts are relatively stable during viral passage; no appreciable loss of the inserts was seen until the fourth viral passage.

One of the problems with recovering shuttle vector DNA after infection is the large amount of contaminating cellular DNA. Unintegrated viral DNA is, in general, easier to recover and clone than integrated DNA, presumably because it is possible to use physical fractionation to enrich for the closed circular forms of unintegrated viral DNA.

We particularly wanted to develop a vector system that makes it simple to recover both unintegrated and integrated DNA. To simplify enrichment of viral DNA, we included the *lacO* sequence in the inserted segment. DNAs containing the *lacO* sequence can be rapidly enriched by binding to *lac* repressor protein (16, 17). The DNA-protein complex is captured on a nitrocellulose filter, and then the DNA is selectively eluted by the addition of IPTG. This simple protocol allowed us to directly clone integration sites. In the initial experiments we used chemically competent cells and were able to get valid clones. However, the cloning of unintegrated viral DNA was more-than-100-fold more efficient when the DNA was introduced into *E. coli* by electroporation.

In addition to recovering integration sites, we want to use these vectors to aid in the recovery of viral vectors that have been adapted by passage. In previous experiments we have found that, in some cases, the initial version of a vector replicated poorly. In some cases, it is possible, with a replication-competent virus, to adapt the vector by passage (1). Variant viruses that grow better have a selective advantage. The adapted virus is obtained simply by repeated passage of the viral stock either in cultured cells or in infected embryos. However, to be used as a vector, the adapted virus must be molecularly cloned and characterized. A shuttle vector should be quite useful in adaptation protocols, provided that the insert is stable enough to be retained during the adaptation process.

The zeocin and blasticidin inserts are stable for three viral passages in the RSVP vectors, even in the absence of drug selection, and should be useful for recovering adapted viruses.

The plasmid cassettes are small and are flanked by *Cla*I sites. It should be possible to introduce these prokaryotic cassettes into other viral vectors. In cases in which the vector will not generate an appropriately spliced message for the selectable markers, the cassette could be linked to an internal promoter or an internal ribosome entry site.

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17. **Pathak, V. K., and H. M. Temin.** 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. Proc. Natl. Acad. Sci. USA **87**:6024–6028.
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20. **Schaefer-Klein, J., I. Givol, E. V. Barsov, J. M. Whitcomb, M. VanBrocklin, D. N. Foster, M. J. Federspiel, and S. H. Hughes.** 1998. The EV-O derived cell line DF-1 supports the efficient replication of avian leucosis-sarcoma viruses and vectors. Virology **248**:305–311.
21. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
22. **Shoemaker, C., J. Hoffman, S. P. Goff, and D. Baltimore.** 1981. Intramolecular integration within Moloney murine leukemia virus DNA. J. Virol. **40**: 164–172.
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EXHIBIT B

Patent
Attorney's Docket No. 00014-002002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Noriyuki Kasahara et al.	Group Art Unit: 1633
Application No.: 10/045,178	Examiner: Ileana Popa
Filing Date: January 11, 2002	Confirmation No.: 7589
Title: A GENE DELIVERY SYSTEM AND METHOD OF USE	

DECLARATION UNDER 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I am not an inventor of the above-identified patent application.

2. I currently do not have any financial or other pecuniary interest in the outcome of this application, the assignee or any licensee.

3. I am currently the Director of Scientific Collaborations at Genentech, South San Francisco, California.

4. I hold the following degrees: University of Maryland, 1974 - B.S. in Pharmacy/Medical Technology; George Washington University, 1979 - M.S. in Biochemistry; George Washington University, 1983 - Ph.D. Genetics, thesis from NIH/NHLBI; Central Michigan University, 1981 - M.A. Health Care Administration. My *curriculum vitae* is attached hereto as Exhibit A.

5. I have been conducting research in the area of recombinant vectors and gene therapy for 24 years, including as a grant receiver and reviewer from 1983-2003 for the NCI and NHLBI in the areas of gene therapy and on various journal review committees and editorial boards. I am currently an inventor of four (4) issued patents and at least eight (8) pending U.S. patent applications related to viral vectors for gene therapy.

6. I am familiar with the work of the inventors of the above-identified application relating to replication competent retroviral vectors. I have also reviewed the pending office action and the specification of the above-identified application. It is my opinion that the vectors of Kasahara et al. as described and claimed in the above-identified application are non-obvious as described below and further that the claimed invention demonstrates unexpected results including stability and transmission that could not have been predicted by those of skill in the art.

7. I am familiar with the work of Ram et al. having collaborated with Ram et al. during the time of the publication of the reference cited in the pending Office Action (see, e.g., reference 18, 20 and 34 listed in my C.V. attached hereto as Exhibit A).

8. It is my understanding that the Examiner believes that the pending claims are obvious in view of the following references applied to the claims: Ram et al. (Cancer Research, 1993, 53:83-88), in view of each of Martuza et al. (U.S. Patent No. 5,585,096), Murakami et al. (Gene, 1997, 202:23-29) and Sobol et al. (U.S. Patent No. 5,674,486). I would respectfully disagree for the following reasons.

The direction/focus of gene therapy vectors at the time that Kasahara et al. developed the invention described in U.S. Application 10/045,178 (the '178 application) were moving away from replication competent retroviral vectors as sources for gene therapy for four (4) reasons: (1) retroviruses were difficult to manipulate due to their ability to accept heterologous genetic inserts, (2) that when heterologous genes were inserted into replication competent retroviruses, it was difficult to maintain stability through repeated passages, (3) increased concern and proposed Federal Regulations related to safety issues in the use of both replication competent and defective retroviral vectors (e.g., the development of lymphomas) in infected individuals, and (4) advances in the development of non-retroviral vectors such as herpes simplex viral vectors, adenovirus vectors and adeno-associated viral vectors, were attractive because of their flexibility and stability. Furthermore, research on replication competent retroviral vectors was, at the time, mainly focused on understanding the viral life cycle and strategies for vaccines or drug inhibitors, particularly as it related to HIV.

Ram et al., as cited by the Examiner, was the prototypical strategy for gene delivery and therapy in 1998 and 1999, i.e., a replication defective vector with reduced or no ability for horizontal or vertical transmission in the absence of a helper viral vector or helper cell. The replication defective systems reduced risk to the subject yet provided a temporary but not long term solution to gene delivery. If anything was to be gleaned from Ram et al. it would have been the ability to make a better replication defective system for gene delivery since this was the direction the technology was proceeding as well as the direction and focus of the laboratory of Ram et al. (note- even as of 2001, Ram et al. was using replication defective vectors, see, e.g., Cohen et al., Isr Med Assoc J., 3(2):117-20, 2001).

It is also important to understand that efforts in gene therapy have focused on attenuating or limiting the replicative capacity or infectivity of retroviral vectors to prevent side effects such as lymphomas. This is why Ram et al., and others, during the early and late 1990s focused on replication defective vectors such as those described above. Even as of 2003, a French gene therapy trial using a defective retroviral vector resulted in a lymphoma in a subject raising concerns about gene therapy. Accordingly, those of skill in the art would be even more hesitant to consider replication competent retroviruses for gene therapy and there was considerable doubt about such vectors.

At the time Drs. Kasahara, Logg and Anderson filed the '178 application and priority application strategies for gene delivery and gene therapy were focused on the development of non-replication competent vectors for gene delivery or focused on DNA- or adenoviral-based vectors. For example, my research in collaboration with Ram et al. focused on replication defective vectors and helper cell systems. Although the research of Ram et al. provided insights into replication defective viral vector systems the research did not provide insight into the development of replication competent systems and more particularly the structural importance of the design of a vector as set forth in the '178 application. Importantly, my understanding of the Ram et al. reference and my understanding through my collaboration with Ram et al. would not have provided a direction to develop replication competent viral vectors based upon Ram et al. in combination with any of the other references.

Other vectors were being researched for gene delivery, such as replication competent Herpes Viral Vectors because they have robust horizontal and vertical infectivity (see, e.g., Martuza et al.). However, the lytic nature of Herpes vectors was not an attractive candidate for gene delivery and long term therapy. A great deal of effort was spent trying to modify the vectors to be less competent. Research on these Herpes-based viral vectors continued to demonstrate that the viral vector became toxic over time. One of skill in the art, such as myself, would not have considered the information and data obtained from a herpes viral vectors as described in Martuza et al., as providing direction for the development of replication competent retroviral vectors as described by Kasahara et al., particularly in view of or in combination with Ram et al. The two vectors are so drastically different that nothing could be developed from one or the other that would relate to the invention of the '178 application. This is simply because the Herpes viral vectors are a different category of vectors (i.e., DNA-based, complex and lytic). It is my understanding that the combination of Ram et al. with Martuza et al. is to provide motivation to develop replication competent retroviruses; however, the Herpes Vectors of Martuza et al. are attenuated to reduce toxicity and the vector's lytic nature. Furthermore, merely indicating the replication competent Herpes viral vectors may be useful does not translate to other vector types nor does it address the numerous technical hurdles (e.g., different genomes, infectivity, life-cycle, cargo capacity of the genome for a gene, and spread through a tissue etc.) related to the development of a replication competent retroviral vector as described in the '178 application. Accordingly, one of skill in the art would not (and could not) have taken the teachings of Ram et al. and Martuza et al. combined them to derive the invention as claimed in the '178 patent.

Other vectors being developed and cited in the Office Action included avian-based viral vectors such as the Rous Sarcoma Virus (RSV). This vector includes a large replaceable gene (*src*) that is unnecessary for the viral life cycle and can be easily modified to be a cargo-carrying domain. Research on RSV does not translate to mammalian systems for one simple reason: the virus cannot infect or replicate in normal mammalian cells (non-genetically modified). Accordingly, the development of an RSV viral vector is an interesting research development, but would not lead one to apply the teachings of RSV to non-avian (e.g., mammalian) retroviral vectors. First, mammalian oncoretroviral vectors do not include a dispensible gene domain that can be manipulated without modifying the transcriptional/translational machinery or size capacity of the vector (see, e.g., Oh et al.,

JOURNAL OF VIROLOGY, Feb. 2002, p. 1762–1768 Vol. 76, No. 4). Second, the vector is useless for treatment of a normal mammalian cell or subject. The combination of Ram et al. with Martuza et al. and Murakami et al. do not provide any insight into the technical features required to obtain the vector of the '178 patent.

Sobol et al. is cumulative to Ram et al. in that both use replication defective vectors in combination with helper cell lines to obtain the desired effect. Sobol et al. teach that a cytokine can be used in the replication defective system. Combining the Sobol et al. reference with the previous references does not teach or suggest, to one of skill in the art, the technical features, the methods of the '178 application, or the compositions of the '178 application. Each of Ram et al., Martuza et al., Murakami et al., and Sobol et al. lend technology to the art in general, however, the combination would not lead one to the advances in gene therapy vectors as set forth in the '178 application.

Taking into account the differences in the genetic makeup and different life cycles of the vectors cited by the Patent Office (i.e., Ram et al., Martuza et al., Murakami et al. and Sobol et al.), I can say with reasonable confidence that one working in the field of viral vector development, such as myself, would not have combined the references as suggested in Office Action. Furthermore, even in the event one of skill in the art would possibly have combined the teachings of the references by (i) piecing genomes together, (ii) changing the lytic nature of one vector type and (iii) overcoming the inability of another vector type to replicate in mammalian cells, the results obtain by Kasahara et al. using the vector described in the '178 application is truly a new thinking of vector delivery and design leading to unexpected results and show great promise in treating various cancers as well as overcoming numerous problems in the art (e.g., stability, transmission, safety).

For example, other attempts at placing heterologous genes into retroviral vectors lead to some short term immediate expression in host cells, however, stability and long term expression was lost in these constructs. The first MLV-based RCR vectors were developed by insertion of either a transgene cassette into the U3 region of the 3' long terminal repeat (LTR), allowing for LTR-driven transgene expression (Dillon, PJ, Lenz, J and Rosen, CA (1991). Construction of a replication-competent murine retrovirus vector expressing the human immunodeficiency virus type 1 tat transactivator protein. J Virol 65: 4490–4493; Stuhlmann, H, Jaenisch, R and Mulligan, RC (1989). Construction and

properties of replication-competent murine retroviral vectors encoding methotrexate resistance. Mol Cell Biol 9: 100–108; Reik, W, Weiher, H and Jaenisch, R (1985). Replication-competent Moloney murine leukemia virus carrying a bacterial suppressor tRNA gene: selective cloning of proviral and flanking host sequences. Proc Natl Acad Sci USA 82: 1141–1145) or an additional splice acceptor site and transgene cassette downstream of the env gene, allowing for transgene expression following alternative splicing (Solly, SK, Trajcevski, S, Frisen, C, Holzer, GW, Nelson, E and Clerc, B et al. (2003). Replicative retroviral vectors for cancer gene therapy. Cancer Gene Ther 10: 30–39). However, these configurations led to genetic instability. In sharp contrast and unexpectedly, the vectors described by Kasahara et al. in the '178 application have improved stability and thus prove to be superior retroviral vectors for therapy. In *in vitro* studies, a small number of RCR vectors according to the '178 application achieved a tremendous amplification of transduced cells, as compared to replication defective retroviral vectors and in one study proved genetically stable when propagated over seven serial cell-free passages (see, e.g., Logg et al., J. of Virol., 75(15):6989-6998, 2001; and Figure 1A-C, below).

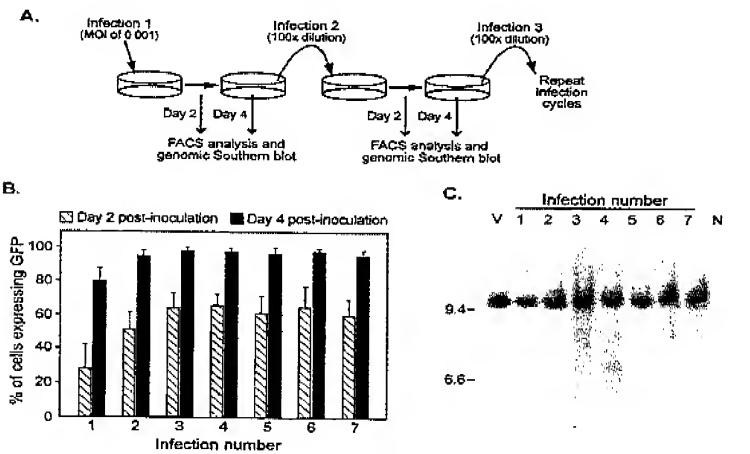


FIGURE 1

Those in the art would not have expected the stability or success of the currently claimed vectors in treating cell proliferative disorders.

As shown in my C.V. I was involved with one of the first Gene Therapy Trials in the World (see reference 25 of my C.V.). Accordingly, I have reviewed a large amount of data and patents related gene therapy trials and *in vitro* experiments. The data demonstrated from Kasahara et al. using the vectors described in the '178 application is truly unexpected and ground breaking. For example, the reference by Tai et al. (Mol. Ther 12:842 (2005)) shows the unexpected efficacy of the invention described by Kasahara et al. in the '178 application (see, Figures 2 and 3, below, depicting the spread and usefulness of the vector ("ACE-CD") of the '178 application).

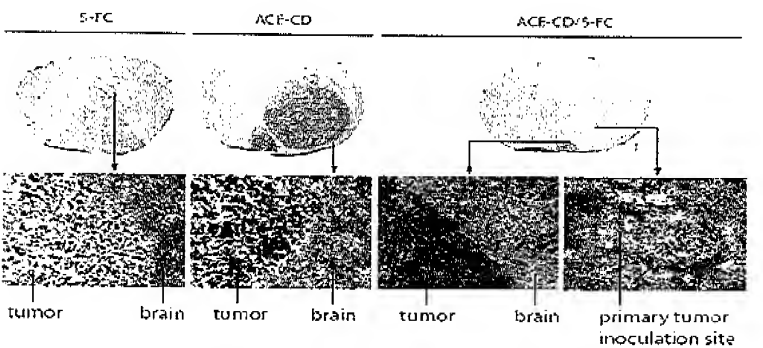


FIGURE 2 (Tai et al. Mol Ther 12:842 (2005))

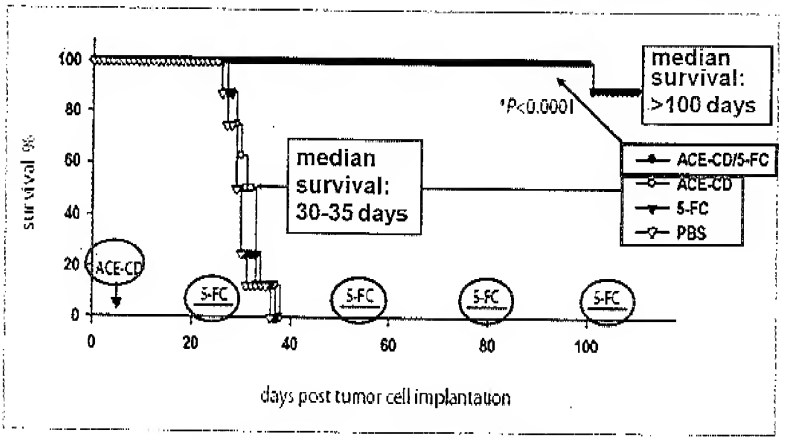


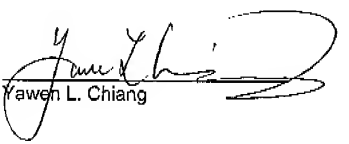
FIGURE 3 (Tai et al. Mol Ther 12:842 (2005))

I am impressed by the ability of the vectors of the presently claimed invention to maintain stability and expression through multiple passages, which at the same time lacking spread to non-cancer tissues/cells. In the field of gene therapy for cancer treatment, the capabilities provided by the currently claimed invention are incredibly important for effective therapy. These capabilities had not previously been obtained and provide a leap and advance in viral vector development. The effective spread and stability of the vectors set forth by the invention were not previously achieved and could not have been contemplated or predicted by the references cited against the presently claimed invention. The field of gene therapy requires innovation such as the invention of the '178 application to move therapy forward in this field. Patent protection is important to protect the hard work and innovative concepts and advance such technology, particularly the innovative contributed by Kasahara et al.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements were made on information and belief and are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: June 11, 2009

By: 
Yawen L. Chiang

CURRICULUM VITAE

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Cell: 6502437568
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Experience

2007-2009

Director, Scientific Collaborations and Contract Management
Genentech /Roche

- Serve as liaison for PR&D with IP Legal and business development: Develop PR&D's strategy for manufacturing technology IP creation and prioritization.
  Maintain CMC-related IP knowledge, track and review the competitive IP landscape.
- Participate in the development of PR&D business development strategy, the evaluation of emerging technologies related to process development, and organize efforts and streamline business processes among the current participants in B/D communications, be the rep for PR&D during the B/D monthly meeting to seek for the proper rep from PR&D for any Due Diligence work.
- Manage the business contracts that support and enable that CMC and PR&D goals are integrated and aligned with the mission and goals of Genentech.
- As CMC Project Leader, responsible on all CMC project needs for Lucentis Long Acting Delivery Program to participate in Program Core Team to represent CMC.

2003-2006

Senior Vice President, Chief Scientific Officer
Corautus Genetics, San Jose, CA

- Responsible for operational management of the California lab.
- Providing strategic and scientific leadership to Corautus to assist in the development of therapeutic angiogenesis product. Using plasmid VEGF2.
- Supervising QC/assay development group, preclinical studies coordination and manufacturing activities to ensure (1) proper technology transfer to collaborators and contractors, (2) appropriate assay development and preclinical studies in alignment with company strategies, (3) proper oversight and interface with manufacturers, and (4) effective communication channels established and implemented for inter and intra departmental activities including communicating with biostatistician, clinical experts and scientific consultants.
- Obtained California State Board of Pharmacy Wholesale Exemption Certificate for Manufacturer of Dangerous Drugs.
- Responsible for FDA regulatory agency communications with CMC, preclinical pharmacology/Toxicology, and clinical reviewers.
- Involved in external funding activities with CEO and CFO.
- Evaluating new technologies for future development.

2001-2003 **Senior Vice President, Research and Development**
GenStar Therapeutics, San Diego, CA

- Responsible for R&D activities, including research teams, manufacturing, process development and quality control programs.
- Strong leadership and management skills in ensuring objectives / milestones are achieved, managing and conducting government grant activities.
- Excellent communication / presentation skills across a wide range of audiences - scientific, FDA regulatory, financial, and Board of Directors.
- Established strong partnership with business / corporate development.
- Work closely with CEO in developing strategy, plans, and timelines for internal and external programs including grants opportunities.

1998-2001 **California Site Director / Senior Director**
Aventis / Gencell (Rhone-Poulenc/Gencell), Hayward, CA

California Site Director

- Site and research head for 70 employee organization focused on oncology, formulation, and preclinical pharmacology/toxicology. Vector development.
- Responsible for Alliance / Collaboration / Project Management and Technology Transfer for worldwide operations in Gene Therapy.
- Directed cancer program - immunotherapy, suicide genes, tumor suppressor genes, antiangiogenesis, and *in vivo* systemic delivery system to target tumor and tumor vasculature.
- Responsible for annual strategic planning and portfolio reviews, for preclinical and technical programs.
- Responsible / accountable for administration services, including HR, purchasing, finance, facilities, IS and worldwide coordination of these functions.

Senior Director, Oncology Gene Therapy

- Provided strategic and scientific leadership to oncology group to identify and develop gene therapy products from concept through preclinical proof of concept in animals.
- Member of the Gencell preclinical research team to develop DI&A strategy for Oncology gene therapy products.
- Managed and coordinated interdepartmental activities to accomplish Gencell's objectives.
- Responsible for scientific collaboration in newly established Aventis / Introgen alliance focused on the development of oncology products using p53. Served as scientific advisory board member.
- Coordinated and communicated with Aventis oncology pharma group and PMC tumor vaccine program.
- Participated in the immunology platform activities and contributed to the global needs for Aventis knowledge management in immunology and oncology.

1993 – 1998 **Vice President, Immunology / Preclinical Studies**
Novartis / GTI (Genetic Therapy, Inc.), Gaithersburg, MD

- Responsible for immunology, assay development, oncology, toxicology and pharmacology.
- Coordinated R&D activities for external activities, including academics, private industry and government institutions.

- Principle Investigator on government grants and NIH CRADA.
- Responsible for preclinical sections of submissions to regulatory agencies.
- International project team for clinical Phase I/II/III studies.
- Support corporate business and legal activities on gene therapy technologies.
- Strong organizational skills, leading integration teams for mergers and acquisitions activities for Novartis, ability to interpret and work effectively within a matrix management system.

1988 - 1993 Director of Immunology

- Conducted research on cancer immuno-gene therapy.
- Directed a laboratory of 24 scientists.
- Participated in the first gene marking and gene therapy trials.

1984 - 1988 **Associate Scientist (Molecular Biology & Immunology)**
Cetus Corporation, Emeryville / Palo Alto, CA

- Work on immunotherapy, concentrated on IL-2 and monoclonal antibodies approaches.
- Provided molecular biology support for the IL-2 project and determined the binding constants of the various IL-2 muteins to the IL-2 receptor using the receptor binding assay.
- Set up experiments for radiolabeling of either ^{35}S-IL-2 or ^{125}I-IL-2.
- Apply the M13 mutagenesis techniques to constructing IL-2 muteins (ala_{125}, thr_{125}, and ala_{104}).
- Project leader for cDNA cloning and expression of immunoglobulin genes, cloning of mouse/human chimeric antibodies, and breast cancer associated antigens identified as immunotoxin targets.
- Development of B cell expression and cloning vector.

1983 - 1984 **Post-doctoral Fellow, Chemist**
Dept. of Pathology, Uniformed Services University of the Health Sciences, DoD.
Advisor: Dr. Esther Chang

- Studied the regulation of oncogene expression as related to cancer induction
- Used Recombinant DNA cloning techniques to study the possible effect of Z-DNAs on cellular transformation.
- Used chromosomal in situ hybridization techniques to sublocalize two human ras pseudogenes.

1979 - 1983 **Graduate student, Chemist, Post-doctoral Fellow**
Molecular Hematology Laboratory, National Institutes of Health
Thesis Advisor: Dr. W. French Anderson

- Studied DNA methylation and regulation of the human β-globin-like genes in mouse/human somatic cell hybrids.
- Performed techniques for the following: tissue cultures; recombinant DNA; DNA transfection; electrophoresis; Southern, Northern and Western blotting; solution hybridization; nucleotides ^{32}P labeling; column chromatography; RNA S_1 mapping; and DNA and RNA direct sequencing.

1976 - 1984 **Supervisor**
Clinical Pathology Lab, Holy Cross Hospital

- Part-time supervisor on weekends in hematology, Clinical Chemistry, Department of Pathology Lab.

1977 - 1978 **Teaching Assistant**
George Washington University
- Lectured in general biochemistry to graduate students and first-year medical students.

1976 - 1978 **Graduate Student, Research Assistant**
George Washington University.
Thesis Advisor: Dr. Linda Gallo
- Studied cholesterol metabolism, "Sublocalization of the Rat Intestinal Cholesterol Esterase.

1974 - 1976 **Medical Technologist** (Full-time),
Pathology Lab, Holy Cross Hospital.

1973 - 1974 **Practical Training**
University of Maryland
- Trained through all sections including chemistry, hematology, microbiology, immunology, virology, immunohematology, histology, coagulation, special chemistry, and special hematology.

Education

2005	Certificate	Preparing the CMC section for NDA's/IND's/CTD's
2005	Training	NIH Angiogenesis: principles and methods
2005	Certificate	ASGT Challenges in Advancing the Field of Gene Therapy: A Critical Review of the Science, Medicine and Regulation Stakeholders'Meeting
2004	Specialized Certificate	Biotechnology Manufacturing, University of California, San Diego Extension
2004	Specialized Certificate	Quality Assurance and Control, University of California, San Diego Extension
2003	Specialized Certificate	Manufacturing Fundamentals for the Drug and Biological Industry, University of California, San Diego Extension
2003	Training Certificate	Outstanding Achievement in Sterile Gowning Practice and the completion of all three Modules for the Current Good Manufacturing Practices for Pharmaceuticals
2002	Course Certificate	Human Participant Protections Education for Research Teams by NIH
2002	Course Certificate	Clinical Gene Transfer Comprehensive review Course by American Society of Gene Therapy
1994	Training Certificate	Biomedical Research Management, Harvard University School of Public Health, Boston, Massachusetts

1983	Ph.D.	Genetics, George Washington University/NHLBI, National Institutes of Health
1981	M.A.	Health Care Administration, Central Michigan University
1979	M.S.	Biochemistry, School of Medicine, George Washington University
1975	Board Certification	Medical Technologist – American Society of Clinical Pathologist (A.S.C.P.)
1974	B.S.	Pharmacy / Medical Technology, Allied Health, University of Maryland, Adelphi, Baltimore, M.D.

Societies:

American Society of Medical Technologists
American Society of Clinical Pathologists
American Association of Advanced Sciences
American Society of Microbiologists
New York Academy of Science
American Associate for Clinical Chemistry
American Society of Gene Therapy
American Society of Hematology

Services:

2002- 2003	National Council for Arts and Sciences, George Washington University
1999- 2002	Industrial Liaison for American Society of Gene Therapy
1994- 2000	NCI,NHLBI,NSF Grant Reviewer, Human Gene Therapy, Cancer Gene Therapy, Molecular gene Therapy journal reviewer, Cancer gene Therapy Editorial Board
1994-1997	Irvington Institute for Immunology, Program Consultant
1983-1984	Assistant Corresponding Secretary, George Washington University, Columbian Women's Association

Honors:

1980 – 1983	George Washington University Scholarship, Department of Genetics
1984	Outstanding Performance Award, Department of Defense, Uniformed Services University of the Health Sciences

Grants:

SBIR Phase I and SBIR Phase II
Interferon Gene Transfer to TIL cells for Cancer
Grant # R44CA53938-02
$500,000 – 2 years (1993-1995)

SBIR Phase I
Upregulation of HSV-TK Gene for Brain Cancer Gene Therapy
Grant#1 R43 CA 68918-01
$99,982 – 6 months (July 1995-January 1996)

PATENTS, APPLICATIONS AND DISCLOSURES:

Issued Patents:

1. **July 20, 1999 Number: 5, 925, 345, "Vectors including foreign genes and negative selection markers." W.F. Anderson and Y. Chiang, et al**
2. **December 7, 1999 Number 5,998,205, "Vectors for Tissue Specific Replication." P. Hallenbeck, Y.Chang and Y. Chiang**

Disclosures:

1. Disclosure No. 87-178-CIC, Patent Committee No. 101, Cetus Corp. "cDNA Clones of Mouse Immunoglobulin Heavy and Light Chain Variable Region from Mouse MAb Anti-C5a (169-10F7)." **Y. Chiang, *R***. Sheng-Dong, and J. Larrick. December 22, 1987.

2. Vector Particles Resistant to Inactivation by Human Serum
   Serial No. 08/326,348
   Filing Date: October 20, 1994; continuation in part of 1992 patent (Anderson & Mason)
   Inventors: W.F. Anderson, **Y. Chiang,** G. McGarrity, and P. Tolstoshev

3. Vectors for Tissue Specific Replication
   Serial No. 08/487,992
   Filing Date: November 28, 1994 and June 7, 1995
   Inventors: Hallenbeck, Chang and **Chiang**

4. Tissue Specific Treatment, Diagnostic Methods, and Compositions Using Replication-Deficient Vectors
   Serial No: 08/348,960
   Filing Date: November 28, 1994 and July, 1996
   Inventors: Hallenbeck, Ramsey, Hammer, and **Chiang**

5. Modified Viral Envelope Polypeptide
   Serial No. 08/409,648
   Filing Date: March 24, 1995
   Inventors: Anderson, Zhao, **Chiang** and MacKrell, Jr.

6. Treatment of Tumors with Cells Expressing Interferons, Tumor Necrosis Factors, or Other Cytokines
   Serial No: 08/431,828
   Filing Date: April 28, 1995
   Inventors: Ennist, **Chiang**, and Forry-Schaudies

7. Gene Therapy of Hepatocellular Carcinoma through Cancer-Specific Gene Expression
   Serial No: 08/444,284
   Filing Date: May 18, 1995
   Inventors: **Chiang**, Hallenbeck, Anderson, and Kaneko

8. Cancer Treatment
   Serial No: 08/527,373
   Filing Date: September 13, 1995
   Inventors: **Chiang** and Chang

9. Viral Vectors Including Polynucleotides Encoding Neurotropic Factors and uses Therefor; GDNF Vectors and Methods of Use
   Serial No: 08/636,548
   Filing Date: April 25, 1996
   Inventors: Bohn, Choi-Lundberg, Lin, Figlewicz, Chang, **Chiang**

10. Prevention o f Graft-Versus-Host Disease with T-cells including

Polynucleotides Encoding Negative Selective Markers
Serial No: 08/656,451
Filing Date: May 31, 1996
Inventors: Munshi, Ennist, Jacob, **Chiang**

11. Optimization of Graft Versus Leukemia (GVL) Reactive Cells
Invention Disclosure, In Progress
Authors: David L. Ennist, William F. Jacob, and **Yawen L. Chiang**

LECTURES:

- John Hopkins University - Taught a biotechnology course on "Development of a New Technology, Gene Therapy" as part of the graduate program. (1994 Fall, 1995 Spring/Summer Semesters and 1996 Spring/Fall Semesters)
- Washington, D.C. -Meeting Chairperson for the international meetings on Gene Therapy: New Technologies and Applications
(April, 1994 and November 1994)
- Cambridge Healthtech Institute, Arlington, VA - Lecture on Gene Therapy: New Technologies and Applications. Topic: "Potential use of IFN for Cancer Gene Therapy"
- London, IBC – International Two-day Conference: "The Treatment of Cancer: Beyond Chemotherapy.
(March, 1995)
- Washington, D.C. – Meeting Chairperson for DNA Technology, Gene Therapy Workshop, Lecture topic: "Characterization of a new improved HSV-TK producing Cell in Cancer Gene Therapy"
(March, 12, 1995)
- Uniformed Services University of the Health Sciences School of Medicine Seminar Series
(February 23, 1996)
- Keynote speaker for the First Annual Conference Chinese Biopharmaceutical Association Mid-Atlantic Region, USA, " Advances in Gene Therapy, "
(April 6, 1996)
- International Gene Therapy Meetings including the first gene therapy meeting at Stanford University invited speaker: "Gene Therapy: Prospects for the Next Decade"
(1997 – 1999)
- Molecular Therapy/Gene Therapy Meeting
Gene Therapy: Science, Application and Ethics Past and Future
(March, 2000)
- Molecular Therapy/Gene Therapy Meeting
Gene Therapy: Immunological Issues
(February, 2002)
- San Diego Gene Therapy meetings
(2002-2003)
- International meeting on Angiogenesis
(November, 20

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2. Gallo L. **Chiang Y. L.**, Vahouny G.V., and Treadwell C. R. Localization and origin of rat intestinal cholesterol esterase determined by immunocytochemistry. *J. Lipid Res.* 21:537-545, (1980)
3. **Chiang Y. L.,** Ph.D. Dissertation: Human Globin Gene Expression in Mouse/Human Somatic Cell Hybrids, George Washington University, May 1983.
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11. **Chiang Y. L.** Direct cDNA cloning and screening of mutants using the polymerase chain reaction. Methods of Enzymology vol 216, Part G, p73-80, Edited by R. Wu, academic Press, (1992).
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References

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EXHIBIT C

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/805,411	05/23/2007	Noriyuki Kasahara	DEHN-024/US	2506

38824 7590 09/01/2009

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EXAMINER	
HILL, KEVIN KAI	
ART UNIT	PAPER NUMBER
1633	

MAIL DATE	DELIVERY MODE
09/01/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of Abandonment	Application No.	Applicant(s)	
	11/805,411	KASAHARA, NORIYUKI	
	Examiner	Art Unit	
	KEVIN K. HILL	1633	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on *17 December 2008*.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 (a) to the final rejection.
   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114).
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☒ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $_____ is insufficient. A balance of $_____ is due.
   The issue fee required by 37 CFR 1.18 is $_____. The publication fee, if required by 37 CFR 1.18(d), is $_____.
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☒ The reason(s) below:

In a telephone conversation with Applicant's representative, Colin Fairman at 612-321-2800 on August 24, 2009, it was confirmed that Applicant let the application go abandoned.

/Anne Marie S. Wehbe/
Primary Examiner, Art Unit 1633

Petitions to revive under 37 CFR 1.137(a) or (b), or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.